**Creating Constructs for Mammalian Cells**

Gibson Isothermal Assembly Strategy (ITA)

Master Stocks required:

1. Tris pH 7.5 (1 M, sterile filtered)
2. MgCl2 (2 M, sterile filtered)
3. dNTP (100 mM dA, dC, dG, dT)
4. dithiotheitol (1 M)
5. oxidized NAD+ (50 mM, from NEB)
6. PEG-8000 (solid)
7. T5 Exonuclease (10U/µL, from Epicentre)
8. Phusion DNA Polymerase (2U/µL, from NEB: avoid Hot Start)
9. Taq Ligase (40 U/µL, from NEB, most expensive reagent)

Working stocks required:

1. T5 Exonuclease (0.2U/µLà 1 µL Tris 7.5, 97 µL H2O, 2 µL master)
2. 5X Isothermal Reaction Buffer (5x IRB) à 1 mL recipe, 120 µL aliquots in -20 °C
    a. 500 µL Tris (1 M)
    b. 25 µL MgCl2 (2M)
    c. 10 µL ea (dATP, dCTP, dGTP, dTTP)
    d. 50 µL DTT (1 M)
    e. 100 µL NAD+ (50 mM)
    f. 250 mg PEG-8000
    g. H2O so Vfinal of 1mL ( ~100 µL)
3. 1.33x Assembly Master Mix (1.3 AMM) à 375µL receipe, 25x 15 µL aliquots in 20 °C
    a. 100 µL 5x IRB
    b. 10 µL T5 Exonuclease (0.2U/µL)
    c. 6.25 µL Phusion DNAP
    d. 50 µL Taq DNA Ligase
    e. 208.75 µL H2O

Other considerations:

1. Primer design – consider number of base pairs of identity in each PCR amplified fragment (30 bp)
2. PCR amplification-gel purify fragments and check concentrations before running a reaction

Protocol for running a reaction:

1. 4x DNA mix (Vtotal= 5 µL for each reaction)
    - use stoichiometric amounts of each fragment - use Duesseldorf's in Silico calculator, 1:1 molar vector to insert ratio
    - dilute to 5 µL using water
2. Incubate at 50° C for 15-60 minutes
3. Transform assembled vectors into desired cell type
    - For electroporation, remember to clean up before transforming

● Plate on LB/Cm(34)